

A Functional Glucocorticoid-Responsive Unit Composed of Two Overlapping Inactive Receptor-Binding Sites: Evidence for Formation of a Receptor Tetramer

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An unusual glucocorticoid-responsive element (called GRE A) was found to mediate the induction of the cytosolic aspartate aminotransferase gene by glucocorticoids and was bound by the glucocorticoid receptor in a DNase I footprinting assay. GRE A consists of two overlapping GREs, each comprising a conserved half-site and an imperfect half-site. The complete unit was able to confer glucocorticoid inducibility to a heterologous promoter (Δ MTV-CAT). Mutation of any of the half-sites, including the imperfect ones, abolished inducibility by the hormone, demonstrating that each of the isolated GREs was inactive. In electrophoretic mobility shift assays, purified rat liver glucocorticoid receptor (GR) formed a low-mobility complex with GRE A, presumably containing a GR tetramer. When purified bacterially expressed DBD was used, low-mobility complexes as well as dimer and monomer complexes were formed. In inactive mutated oligonucleotides, no GR tetramer formation was detected. Modification of the imperfect half-sites in order to increase their affinity for GR gave a DNA sequence that bound a GR tetramer in a highly cooperative manner. This activated unit consisting of two overlapping consensus GREs mediated glucocorticoid induction with a higher efficiency than consensus GRE.

Steroid hormones regulate gene expression by interacting with intracellular receptors which, in turn, bind to specific DNA sequences and modulate the transcription of the target genes (6, 16). These receptors belong to a family of proteins that includes ligand-dependent transcriptional regulators as well as proteins for which no endogenous ligands have been identified (30). On the basis of the primary structure of these proteins and the sequence of the DNA-responsive elements, two classes of receptors have been identified: (i) receptors for estrogens, thyroid hormones, vitamin D, and retinoic acid as well as several orphan receptors (11) and (ii) receptors for glucocorticoids, mineralocorticoids, progestins, and androgens (6).

During the last few years, a considerable amount of work has been devoted to identifying the DNA sequences recognized by these receptors. A consensus (half-site) sequence has been found for each class of receptor: TGACC for the estrogen receptor/thyroid hormone receptor subfamily and GTTCT for the glucocorticoid receptor (GR) subfamily (2, 4, 39). It is believed that each of these sequence motifs binds one receptor molecule. Since receptors optimally bind to DNA as dimers, efficient hormone-responsive elements consist of two of these conserved sequence motifs (or half-sites) (36).

The arrangement of these half-sites is at least as critical for efficient binding as the DNA sequence itself. In the estrogen-responsive element (ERE), half-sites are arranged as inverted repeats separated by 3 bp (25). The analysis of several promoters regulated by other receptors of this subfamily suggests that responsive elements are not frequently arranged as inverted repeats. In fact, several receptors of this subfamily, i.e., thyroid

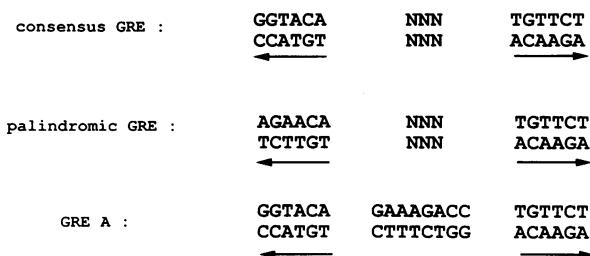
hormone receptor, vitamin D receptor, and retinoic acid receptor, form heterodimers with retinoid X receptor and exhibit a high affinity for direct repeats of the half-sites (21, 25, 41, 42). The number of base pairs separating these direct repeats is a critical determinant of binding selectivity for each receptor. An interesting recent finding is that these receptors are capable of binding to both elements with inverted repeats and elements with direct repeats (19, 29).

All receptors of the GR subfamily bind with high affinity to the same element composed of inverted repeats separated by 3 bp (2). In fact, the consensus sequence, called the glucocorticoid-responsive element (GRE), is not a perfect palindrome (Fig. 1A). These observations raised two important questions: How can a single element account for distinct effects of different hormones? How can a single element explain the different responses to glucocorticoids of different genes? Recent observations suggest that the presence of multiple adjacent hormone-responsive elements and their proximity to binding sites for transcription factors may provide an answer to some of these questions (reviewed by Truss and Beato [36]). Clearly the arrangement of the hormone-responsive elements within the promoter is critical. Cooperative binding of two receptor dimers to adjacent sites has also been described in several cases (23, 33, 34, 38). In one recent study, the binding of a GR trimer to the POMC-negative GRE raised the possibility that complex GRE could be recognized by unusual GR multimers which could display specific regulatory properties (9).

During our study of the glucocorticoid regulation of cytosolic aspartate aminotransferase (cAspAT), we found an unusual GRE-like sequence composed of two conserved half-sites arranged as an inverted repeat separated by 8 bp instead of 3 (28) (Fig. 1A). Here, we analyze the properties of this unusual element and show that it is functional and is composed of two overlapping GREs, each consisting of a high-affinity half-site

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A.



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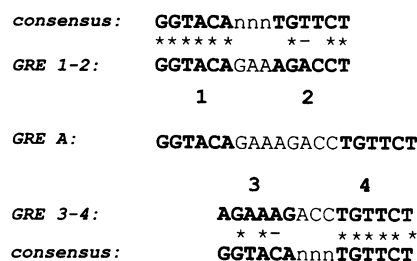


FIG. 1. (A) Sequences of consensus GRE, palindromic GRE, and GRE A. (B) The 20-bp sequence of GRE A. GRE 1-2 and GRE 3-4 correspond to the 15 bp at the left and right sides, respectively, of the GRE A sequence. Stars indicate identical bases, and minus signs indicate critical mismatches.

and a low-affinity half-site separated by 3 bp. The entire sequence probably binds two GR dimers cooperatively.

MATERIALS AND METHODS

Cell culture. The human hepatoma cell line HepG2 (17) was cultured under the conditions described by Pavé-Preux et al. (28).

Plasmids. The rat GR expression vector RSV-GR (24) was a generous gift of Keith Yamamoto (San Francisco, Calif.). Plasmid Δ MTV-CAT was derived from plasmid MTV-CAT by deletion of the sequence from position -190 to -88 of the mouse mammary tumor virus long terminal repeat. It was a kind gift of Ron Evans (San Diego, Calif.), and its construction was described by Umesono et al. (40). A *Hind*III site was created at the deletion site and used as a cloning site for all oligonucleotides used in this study. The double-stranded oligomers (Fig. 2) all have 5' extensions that are coherent with a *Hind*III site. However, the restriction site is lost in the recombinant plasmids.

Transfection experiments. HepG2 cells were transfected as described by Garlatti et al. (13), with some modifications. Five micrograms of the reporter plasmid was cotransfected with various amounts (0 to 100 ng) of a GR expression vector (RSV-GR). Following transfection, cells from two dishes were detached by trypsin treatment, mixed, and seeded equally into two new dishes. Dexamethasone (0.1 μ M) was added 24 h later to one of the dishes. This procedure was devised to ensure equivalent transfection efficiency in control and treated cells. After a 24-h treatment, cells were homogenized for chloramphenicol acetyltransferase (CAT) assay.

CAT assay. CAT activity was determined by using the two-phase assay developed by Neumann et al. (26). Briefly, cell homogenates were prepared in 280 μ l of 250 mM Tris-HCl

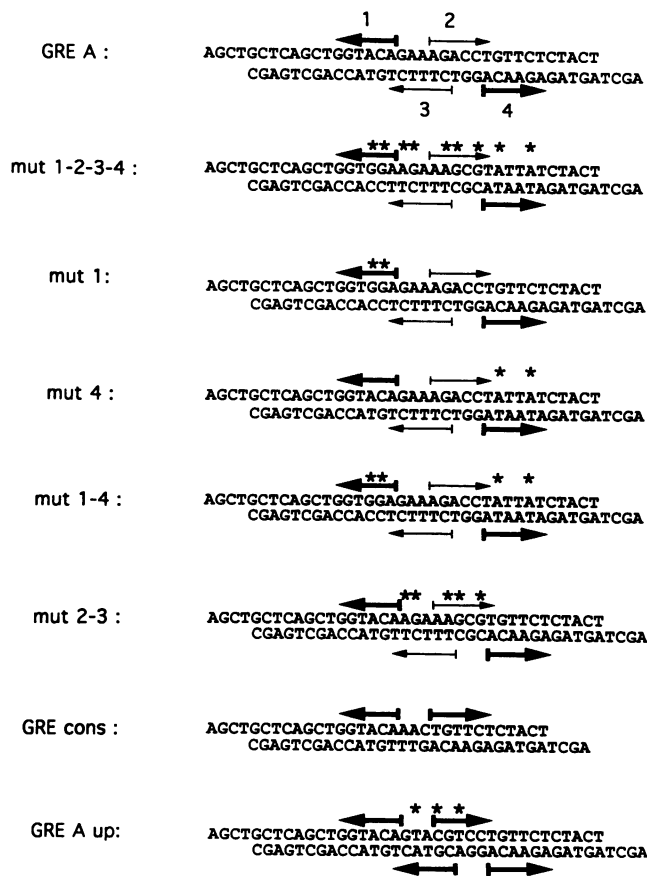


FIG. 2. Full-length sequences of the oligonucleotides used in this study. Thick arrows represent conserved half-sites, and thin arrows represent low-affinity half-sites. cons, consensus.

(pH 7.8)–5 mM EDTA as described previously (15). Cell extracts (80 μ l) were treated at 65°C for 5 min, in order to inactivate the endogenous acylases, and then centrifuged at 12,000 rpm for 15 min. For each reaction, 20 μ l of the cell extract supernatant was added to 40 μ l of a buffer solution in order to have the following final concentrations: 250 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM chloramphenicol, 30 μ M acetyl coenzyme A (acetyl-CoA), and 0.5 μ Ci of [3 H]acetyl-CoA (NEN product no. NET-290 L). The reaction was carried out for 30 min at 37°C. The solution was then transferred to a minivial and layered with 4 ml of Econofluor (NEN product no. NEF 969). After vigorous mixing, the two phases were allowed to separate for at least 15 min, and then radioactivity was counted in a scintillation counter. Under these conditions, the product of the reaction, acetylated chloramphenicol, but not the unreacted acetyl-CoA, is allowed to diffuse into the Econofluor phase. In these experiments, blanks are obtained by assaying CAT activity in cells that have undergone the same treatment in the absence of a CAT plasmid. Proteins were assayed by the method of Bradford (3).

Receptor preparation. Rat liver GR was purified as previously described (14). The DNA-binding domain (DBD) of the GR was expressed in *Staphylococcus aureus* as a fusion protein and purified as described previously (5). Recombinant full-length human GR was expressed in *Spodoptera frugiperda* Sf9 cells by using the baculovirus expression vector system and was isolated as described by Srinivasan and Thompson (35).

DNase I footprinting. The p(-553, -26) CAT plasmid (13) was linearized at the *Bam*HI site, just upstream of the cAspAT gene promoter fragment. Following dephosphorylation, it was 5' end labeled with [γ -³²P]ATP and T4 polynucleotide kinase by established procedures (22). After a second cut with *Xho*I (the downstream cloning site of the promoter fragment), the purified, labeled fragment (40,000 cpm) was incubated with 500 ng of rat liver GR in 200 μ l of a buffer containing 10 mM Tris-HCl (pH 7.5), 0.1 mM dithiothreitol, 0.1 mM EDTA, 10% (vol/vol) glycerol, and 100 mM NaCl for 45 min at 25°C, after which 10 U of DNase I, 0.5 μ g of poly(dA-dT), and MgCl₂ to a final concentration of 5 mM were added and allowed to react for 30 s before the reaction was stopped with EDTA, phenol-chloroform extraction, and ethanol precipitation. The digested DNAs were then loaded on a 6.5% sequencing gel as previously described (37).

Electrophoretic mobility shift assay (EMSA). The sequences of the oligonucleotides used are shown in Fig. 2. The length of the sequence flanking the wild-type or mutated GRE A (at least 9 bp) was chosen in order not to decrease the affinity of the receptor for the oligonucleotide (7). Oligonucleotides were hybridized and labeled by using the Klenow fragment of DNA polymerase I as described by Garlatti et al. (13). The assay was done essentially as described elsewhere (5). Binding reactions were carried out in a 20- μ l volume containing 20 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 1 mM EDTA, 10% (vol/vol) glycerol, 3 μ g of bovine serum albumin per μ l, 100 mM NaCl, 0.3 ng of radiolabeled purified DNA probe, and 10 or 100 ng of calf thymus DNA in the case of DBD and 100 ng in the case of GR. In some experiments, 0.1% Nonidet P-40 was added to the medium, to minimize the binding of accessory protein to GR (18), with no change in the binding pattern. GR at the amounts indicated in the figure legends was added last. After incubation at room temperature for 15 min, the reaction mixtures were loaded on a preelectrophoresed (100 V/12 cm, 60 min) 4% polyacrylamide gel (acrylamide/bisacrylamide, 40:1) containing 0.5 \times Tris-borate-EDTA, and electrophoresis was continued for 90 min. Gels were then dried and autoradiographed. In supershift experiments, antibodies (0.1 mg/ml) were incubated with the receptor on ice for 2 h prior to the binding reaction. Monoclonal antibody BuGR₂ was from Affinity Bioreagents and the control myeloma immunoglobulin G2 was from Sigma.

Methylation interference assay. The assay was carried out as described by Truss et al. (37). Briefly, end-labeled oligonucleotides were treated with dimethyl sulfate (DMS) in a Maxam-Gilbert G reaction (22). The methylated DNA was then added to a binding reaction mixture with 7 or 13 μ l of GR preparation or 5 μ l of DBD preparation, in a final volume of 40 μ l, as described above. The reaction mixture was electrophoresed through a 4% polyacrylamide gel, which was transferred to a DEAE-cellulose membrane (DE81; Schleicher & Schuell). This was followed by autoradiography, excision of the bands, and elution overnight at 37°C in 300 μ l of Tris-EDTA buffer containing 1.5 M NaCl. After chloroform-isoamyl alcohol extraction and ethanol precipitation, the DNA was cleaved with piperidine as described previously (22) and subsequently electrophoresed on a 15% acrylamide-8 M urea gel, which was dried and autoradiographed.

RESULTS

We have previously shown that a 2.4-kb fragment of the cAspAT gene promoter displays a hormonal regulation similar to that of the endogenous gene in Fao rat hepatoma cells (1). In Fao cells stably transfected by this promoter fragment

preceding the CAT gene, glucocorticoids induced the promoter activity whereas insulin inhibited and cyclic AMP potentiated the glucocorticoid effect. Deletion of this promoter fragment revealed that maximal glucocorticoid induction required two sites, a distal one located between bp -1983 and -1718 and a proximal one located within the 656 proximal bp. Further deletion of the latter fragment showed that the proximal GRE was located within the fragment from -553 to -398 (fragment -553/-398) (data not shown).

Binding of the GR to GRE A. Sequence analysis of fragment -553/-398 revealed two hexamers, GGTACA and TGTCTC, located at positions -462 to -454 and -446 to -440 (28). The sequence of the entire site, referred to as GRE A, is shown in Fig. 1A. This site includes two typical GRE half-sites arranged as an inverted repeat separated by 8 bp. As mentioned, a consensus GRE has a similar arrangement except that the half-sites are separated by 3 bp (Fig. 1A).

We first examined whether GRE A could bind the purified GR. Figure 3 shows a DNase I footprinting experiment using fragment -553/-26 of the cAspAT gene promoter in the presence or the absence of the GR. One site was shown to be protected by the GR; it covered the sequence -464 to -431, which includes GRE A with additional base pairs on each side. The limits of the footprint are the two hypersensitive sites which are indicated in Fig. 3. The data showed clearly that the GR efficiently bound to GRE A. Interestingly, another conserved half-site located at positions -382 to -377 was not protected from DNase I digestion, suggesting that under the experimental conditions used, the presence of one conserved half-site was not sufficient to promote stable binding of the GR.

At this stage, two models could account for our observations. The first model predicts that a dimer would bind to GRE A, with each monomer interacting with the highly conserved half-sites. In this case, the distance separating the GR monomers would be 5 bp longer than the distance separating them in the consensus GRE, which means that the monomers would be on opposite sides of the DNA B helix. The second model predicts that two GR dimers would bind to overlapping GREs (GRE 1-2 and GRE 3-4), each composed of a conserved (high-affinity) half-site and a less specific (low-affinity) one, 3 bp apart (Fig. 1B). The low-affinity half-sites would be contained within the 8-bp linker sequence. For simplicity, we refer to this complex with one GR dimer on each side of the GRE A double helix as a GR tetramer. The following experiments were devised to distinguish between these two models.

EMSAs were conducted with either palindromic GRE (GRE pal, in which the high-affinity half-sites are separated by 3 bp [37]) or GRE A in the presence of increasing concentrations of bacterially produced GR DBD (5). In the case of GRE pal, an abundant complex corresponding to the DBD dimer appeared at the lower concentration used (Fig. 4A). A faint band corresponded to the complex formed with the DBD monomer. At the highest concentration used, all of the probe was associated with the DBD dimer, and even under these conditions, no lower-mobility complex appeared. When GRE A was used as the substrate, no significant complexes were observed at the lower concentration, but four retarded complexes (bands 1 to 4) were observed at high DBD concentrations (Fig. 4A). Bands 1 and 2, which display the highest intensities, migrate as complexes containing DBD monomer and dimer, respectively. Bands 3 and 4 could correspond to complexes containing additional GR DBD molecules (possibly trimer and tetramer, respectively) or, alternatively, to complexes with a different conformation but no additional GR

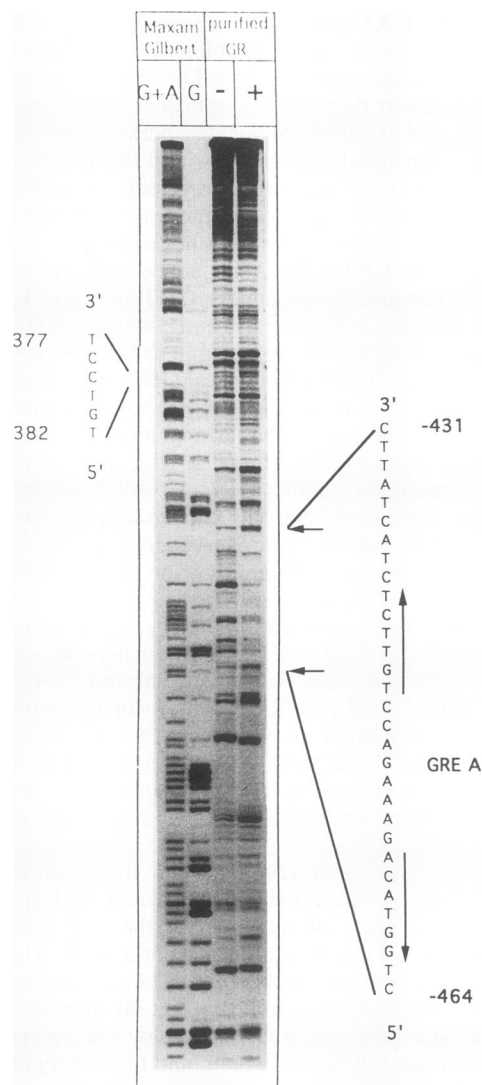


FIG. 3. DNase I footprinting. Purified rat liver GR (500 ng) was incubated with the cAspAT gene promoter probe, and DNase I footprinting was carried out as described in Materials and Methods. The two first lanes show purine-specific (A+G) or guanine-specific (G) sequence reactions. The DNase I reaction was carried out following an incubation with (+) or without (−) GR. The protected region between −464 and −431 is indicated, and the sequence within this region is given. Another region (−382 to −377) containing a GRE half-site is also indicated.

DBD molecules. All complexes were displaced by an excess of unlabeled GRE A or GRE pal (data not shown).

A similar experiment was conducted with purified rat liver GR (Fig. 4B and C). Retarded complexes were obtained with both GRE pal and GRE A, but these complexes displayed distinct mobilities. In the case of GRE pal, the GR bound to DNA both as a monomer and as a dimer at low receptor concentrations and mostly as a dimer at high concentrations. The relative abundance of the monomer form of the receptor complex could be due to the small amount of GR used or to the dissociation of the dimer during migration of the gel. Alternatively, this band could be due to the binding of another rat liver cytosolic protein, an unlikely explanation in view of the supershift experiments (see below). In contrast, the GR

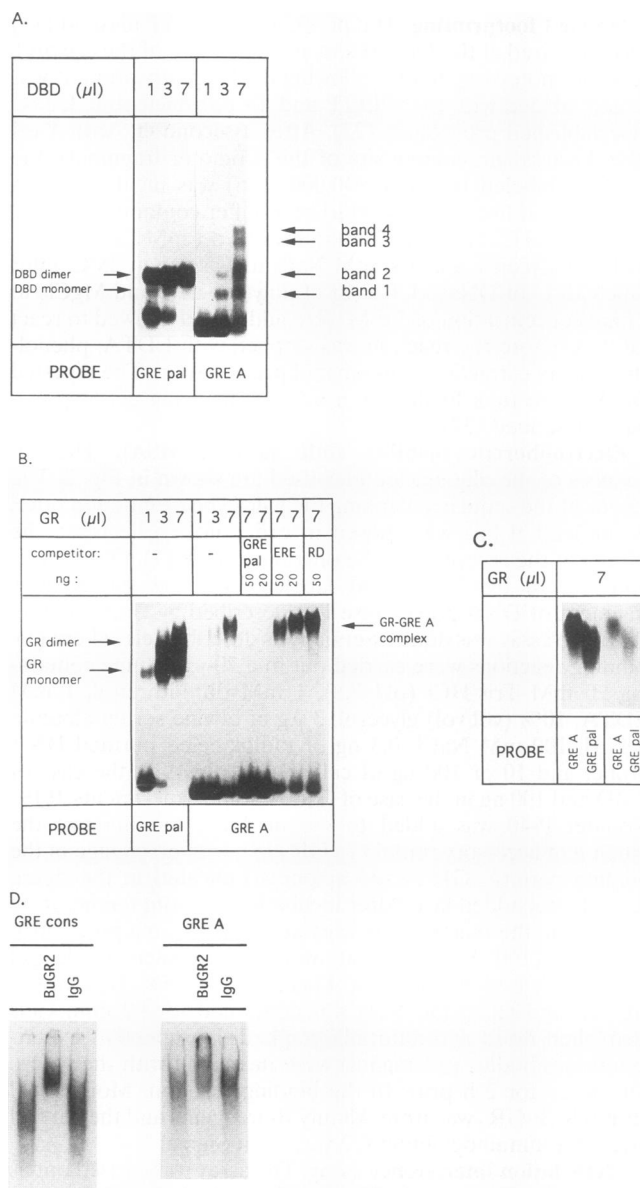


FIG. 4. EMSA with DBD and GR. (A) Binding to DBD. Increasing amounts (1, 3, and 7 μl) of a partially purified preparation of the GR DBD expressed in *S. aureus* were incubated with labeled oligonucleotides corresponding to GRE pal or GRE A. Positions of migration of the DBD monomer and dimer deduced from GRE pal binding are indicated to the left. Positions of migration of bands 1 to 4 obtained with DBD binding to GRE A are indicated to the right. (B and C) Binding to GR. (B) Increasing amounts (1, 3, and 7 μl) of a purified rat liver GR preparation were incubated with labeled GRE A and GRE pal. GR monomer- and GR dimer-containing complexes are indicated to the left. The novel GR-GRE A complex is indicated to the right. Reactions in panels A to C were carried out in the presence of 100 ng of calf thymus DNA. When indicated, 20 or 50 ng of competitor oligonucleotide was added. ERE is an oligonucleotide containing a high-affinity binding site for the estrogen receptor (5), and RD is an oligonucleotide of the same size as GRE pal and ERE with a random sequence. (C) Similar experiment using 7 μl of the GR preparation in which the GR-GRE A complex and GR-GRE pal complex (GR dimer) migrated side by side to show the difference in migration. A short exposure of the same gel is shown at the right. (D) Supershift assay. Rat liver GR was preincubated for 2 h at 4°C in the absence or presence of the BuGR₂ or immunoglobulin G2 antibody and then incubated with the GRE consensus or GRE A oligonucleotide as described above.

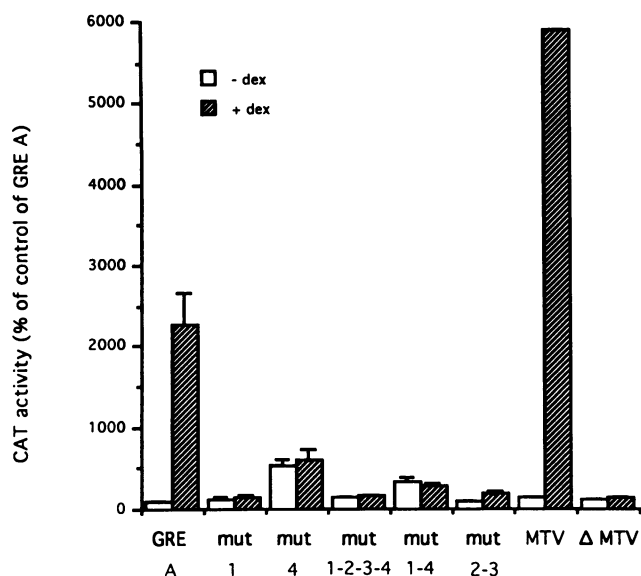


FIG. 5. Effects of half-site mutations on the activity of GRE A. The sequences of the various wild-type or mutant oligonucleotides are shown in Fig. 2. Oligonucleotides were subcloned in plasmid Δ MTV-CAT. Each construct (5 μ g) was cotransfected with 30 ng of RSV-GR in HepG2 cells. MTV-CAT and Δ MTV-CAT were used as positive and negative controls, respectively. Cells were untreated or treated with 0.1 μ M dexamethasone (dex). CAT activity was determined and expressed as a percentage of the activity of cells transfected with GRE A- Δ MTV-CAT and not treated with dexamethasone. Each point is the average \pm standard error of three independent determinations.

formed a complex with GRE A that displayed a slower mobility than the dimer (Fig. 4B and C) at intermediate and high receptor concentrations. Under these conditions, the intensities of the bands that could correspond to receptor monomer or dimer were very weak irrespective of the receptor concentration. No complexes were formed in the presence of an excess of unlabeled GRE pal oligonucleotide. Addition of an excess of unlabeled ERE or random oligonucleotide did not prevent complex formation. Although no detailed kinetic analysis was conducted, Fig. 4 shows that the apparent affinity of GR for GRE A is approximately threefold lower than its affinity for GRE pal.

To confirm that the low-mobility complex is a GRE A-GR complex, the following experiments were carried out. Recombinant full-length GR produced from baculovirus-infected Sf9 cells was tested and gave essentially the same results as rat liver GR (not shown). Furthermore, monoclonal antibody BuGR₂ supershifted the rat liver GR-GRE A complex as well as the rat liver GR-consensus GRE complexes, confirming the presence of GR in these complexes (Fig. 4D). Another interesting observation is that in the case of the GR DBD, several complexes were observed with GRE A even at high concentrations, whereas in the case of the GR, only one major complex was formed.

Functionality of GRE A. We next examined whether GRE A was functional in the context of a heterologous promoter. Plasmid Δ MTV-CAT consists of the CAT gene driven by the long terminal repeat of mouse mammary tumor virus from which fragment -190/-88, including the hormone regulatory sequences, was deleted (40). The CAT activity of cells transfected with this plasmid was not induced by glucocorticoids, while that of cells transfected by plasmid MTV-CAT was

highly inducible (Fig. 5). An oligonucleotide containing the GRE A sequence was subcloned in the *Hind*III site of plasmid Δ MTV-CAT (Fig. 2). The recombinant plasmid GRE A- Δ MTV-CAT was cotransfected with 30 ng of plasmid RSV-GR, an expression vector for the GR, into HepG2 cells. As shown in Fig. 5, dexamethasone induced CAT activity up to 20-fold under these conditions. These data show that GRE A can mediate the activation of transcription of a heterologous promoter by the GR and thus constitutes a bona fide inducible transcriptional enhancer.

Mutations of GRE A. Experiments using mutated oligonucleotides were conducted to determine the contribution of each putative half-site to the transcriptional regulation mediated by GRE A. The highly conserved half-sites (half-sites 1 and 4; Fig. 2) were modified by replacement of the most critical bases for GR binding (27). The less conserved half-sites (half-sites 2 and 3) were mutated by replacement of the few bases that were homologous to the consensus GRE sequence. The remaining sequence, including that of the flanking regions, was left unchanged. The modified oligonucleotides (Fig. 2) were subcloned in the *Hind*III site of plasmid Δ MTV-CAT, and the resulting constructs were cotransfected with 30 ng of plasmid RSV-GR in HepG2 cells. These constructs were named according to the inactivated half-site (Fig. 2). We first verified that in cells transfected with the mut 1-2-3-4 (in which all four half-sites were mutated) Δ MTV-CAT plasmid, CAT activity was not regulated by dexamethasone even when RSV-GR was cotransfected with the reporter plasmid (Fig. 5). This finding indicates that the regulation observed with the GRE A- Δ MTV-CAT construct is indeed mediated by at least one of the GRE-like sequences in this oligonucleotide. We next examined the effect of inactivating either one of the conserved half-sites or both together (mut 1, mut 4, and mut 1-4; Fig. 5). In all cases, the regulation of CAT activity by dexamethasone was completely lost. In the case of mut 4, basal activity was higher than in the other cases, but no further increase was observed upon dexamethasone addition. We conclude that the integrity of both conserved half-sites is required for GRE A-mediated glucocorticoid regulation.

The contribution of the less conserved half-sites, 2 and 3, was then evaluated by replacement of five bases located between the two conserved half-sites, 1 and 4. In the resulting oligomer, mut 2-3, the 8-bp sequence separating the two conserved half-sites displays no homology to a GRE half-site (Fig. 2). In cells transfected with this construct, CAT activity was induced only twofold by dexamethasone (Fig. 5). This small effect was reproducible but was more than 10-fold less than the effect obtained with the GRE A construct. Thus, the poorly conserved half-sites, 2 and 3, are required for efficient regulation by dexamethasone. These data argue against a model whereby GR binds only to half-sites 1 and 4 without interacting with the 8-bp intervening sequence.

In addition to this functional analysis, the pattern of binding of the DBD to the various mutant oligonucleotides was studied. High concentrations of the DBD were added to the various probes in the presence or absence of an excess of unlabeled consensus GRE oligonucleotide. One major complex corresponding to the DBD dimer was formed with consensus GRE (Fig. 6). While the expected four complexes were obtained with GRE A as the substrate, the mut 1-4 and mut 1-2-3-4 oligonucleotides yielded no detectable complexes (Fig. 6). In the case of mut 1, a faint band corresponding to a DBD monomer-containing complex could be detected. The same complex could be detected with mut 2-3, but it was significantly more abundant. A faint band corresponding to a DBD dimer-DNA complex could also be detected in this case.

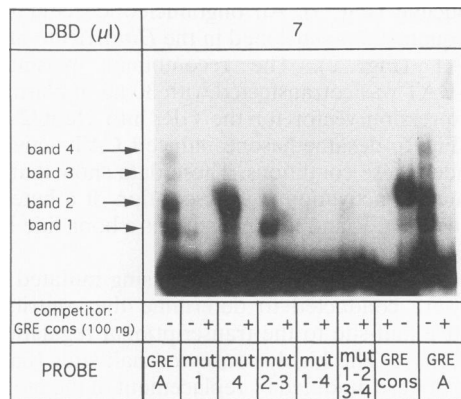


FIG. 6. Binding of DBD to mutant oligonucleotides. The mutants used in the functional assay shown in Fig. 5 were tested for the ability to bind the DBD in an EMSA. The conditions for labeling the probes, binding to the DBD, and migration were as described in Materials and Methods. For each oligonucleotide, the binding assay was performed in the absence or presence of 100 ng of consensus GRE (GRE cons). In each case, 7 μ l of the DBD preparation was used.

Finally, the mut 4 DNA formed a stable complex with a DBD dimer. Thus, the pattern of binding of DBD to the various mutant oligonucleotides depends on the type of mutation. However, in all cases, the slowly migrating complexes detected with wild-type GRE A and referred to as bands 3 and 4 were not detected with any of the mutants. Thus, the presence of these retarded complexes correlates with the functional activity of the GRE A sequence. This property is specific to GRE A, since in the case of consensus GRE, it is the presence of DBD as a dimer in the complex (band 2) that correlates with functional activity. The formation of such a dimer with one of the GRE A mutants, mut 4, is obviously not sufficient to confer glucocorticoid responsiveness to the resulting sequence. A similar experiment was conducted with purified rat liver GR instead of the DBD. Similar results were obtained: a diffuse band corresponding to the GR dimer complex was detected with mut 4 and to a lesser extent with mut 2-3. In the case of the other mutants, no retarded complexes corresponding to GR monomer or dimer or to a GRE A-GR complex were detected (data not shown). The conclusions of the mutational analysis are that a GRE A with a single GR dimer is not functional and that two GR dimers are required for activity. This strong functional synergism between GR dimers suggests the formation of a GR tetramer.

Activated GRE A. The foregoing data are compatible with our second model, whereby the GRE A-GR complex is composed of two GR dimers binding to overlapping sites. Each site includes one high-affinity half-site (1 or 4) and one low-affinity half-site (2 or 3). One prediction of the model is that by modifying half-sites 2 and 3 so that critical bases for GR binding are included, the affinity of the resulting sequence for GR will be increased. We have synthesized such a sequence, which consists of two overlapping elements, each similar to a consensus GRE (GRE A up; Fig. 2). The distance between the center of each GRE in this sequence is 5 bp or half of a DNA helix turn. Functional and binding analyses were carried out with this novel sequence.

When subcloned in plasmid Δ MTV-CAT, the GRE A up construct mediated a potent glucocorticoid induction of CAT activity even in the absence of added GR expression vector (Fig. 7). In fact, the cotransfection of such an expression vector did not increase the dexamethasone effect significantly, sug-

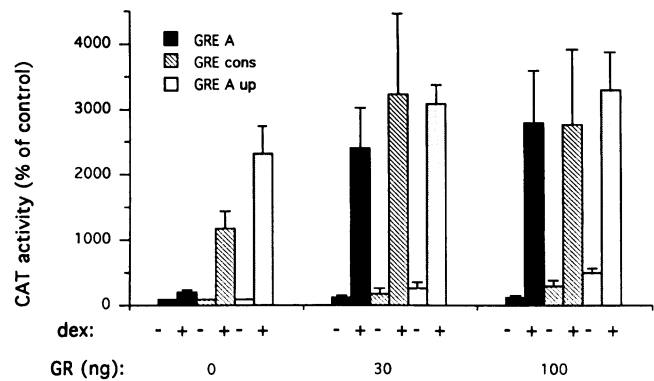


FIG. 7. Effects of base substitutions activating the GRE A oligonucleotide. Specific bases in half-palindromes 2 and 3 of GRE A were substituted, yielding oligonucleotide GRE A up. The results of a functional assay similar to the one shown in Fig. 5 are presented. The oligonucleotides were subcloned in plasmid Δ MTV-CAT, and the resulting construct (5 μ g) was cotransfected with RSV-GR in HepG2 cells. CAT activity was determined in cells untreated or treated with 0.1 μ M dexamethasone (dex) and was expressed as a percentage of control activity. One hundred percent represents the activity of cells transfected with the reporter plasmid alone and not treated with dexamethasone. Each point is the average \pm standard error of three to four independent determinations. cons, consensus.

gesting that the amount of endogenous receptor was sufficient. In comparison, the consensus GRE-mediated effect was increased 2-fold in the presence of cotransfected plasmid RSV-GR, and the GRE A-mediated effect was increased 10-fold under these conditions. These data suggest that GRE A up

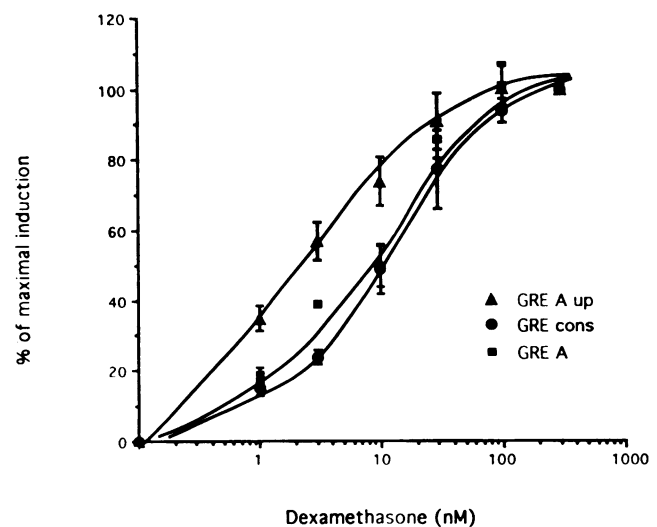


FIG. 8. Dose-response curve of the induction of CAT activity by dexamethasone. HepG2 cells were transfected with 5 μ g of either GRE A- Δ MTV-CAT, GRE cons- Δ MTV-CAT, or GRE A up- Δ MTV-CAT in the presence of 10 ng of RSV-GR. All cells transfected with the same reporter plasmid were pooled and seeded at the same number in different dishes. One day later, increasing concentrations of dexamethasone were added for 24 h, and CAT activity was determined. Data are expressed as a percentage of maximal induction for each curve. Each point is the average \pm standard error of three independent determinations.

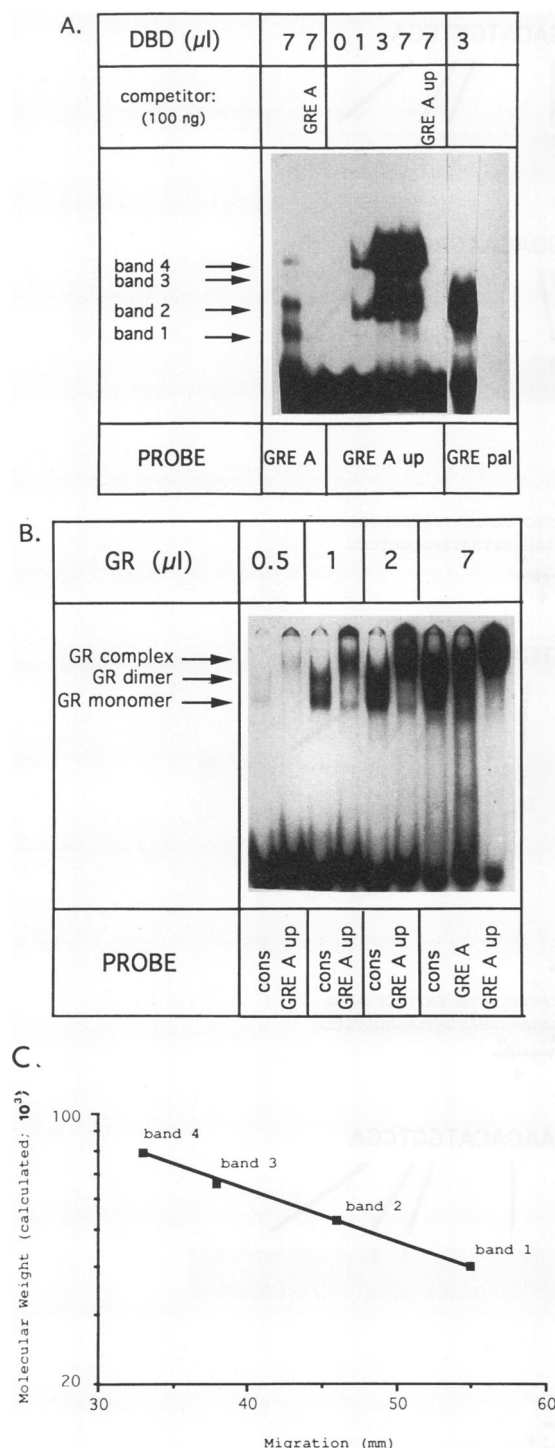


FIG. 9. Binding of GR and DBD to GRE A up revealed by EMSA. (A) GRE A, GRE A up, and GRE pal were labeled and incubated with DBD as described in Materials and Methods. When indicated, incubation was done in the presence of 100 ng of unlabeled oligonucleotide. Different amounts of DBD (1, 3, and 7 μ l) were used with GRE A up (compare with Fig. 4A). Migration of bands 1 to 4 is indicated to the left. (B) Increasing amounts of a GR preparation were incubated with the same amount of labeled consensus GRE or GRE A up. Following the binding reaction, bound and free probes were separated on an acrylamide gel. A binding reaction with the highest amount of GR was also performed with GRE A. Positions of migrations of GR monomer and GR dimer (deduced from consensus GRE

constitutes a very potent glucocorticoid-responsive unit. Similar data were obtained with NIH 3T3 cells (not shown).

Because of the unusual structures of GRE A and GRE A up, we hypothesized that the pattern of response to GR activation may be different from that of consensus GRE. HepG2 cells were transfected with 10 ng of RSV-GR with either plasmid GRE A- Δ MTV-CAT, GRE cons- Δ MTV-CAT, or GRE A up- Δ MTV-CAT. Different concentrations of dexamethasone were added to the cells, and CAT activity was measured. Dose-response curves are shown in Fig. 8. There was no clear difference between the half-maximal induction obtained for GRE A and consensus GRE (approximately 10^{-8} M). However, in the case of GRE A up, the dose-response curve was shifted to the left. Half-maximal induction was attained at 2×10^{-9} M.

Binding of recombinant DBD and of rat liver GR to GRE A up was analyzed by EMSA. As shown in Fig. 9A, two abundant complexes were formed upon addition of increasing concentrations of DBD to GRE A up. One complex migrated as band 2 and probably corresponds to DBD dimer. The second complex migrated as band 4 but was much more abundant than the corresponding complex obtained with GRE A. The most likely interpretation is that this complex corresponds to a GR tetramer resulting from the binding of a GR dimer to each of the two conserved GREs constituting GRE A up. The notion that band 4 corresponds to a tetramer complex is consistent with a plot of the mobilities of the various complexes (bands 1 to 4) versus their molecular weights, assuming that they contain one to four DBD molecules, respectively (12). As shown in Fig. 9C, this plot shows a linear relationship. The tetramer complex with GRE A up was detected at the lowest concentration of DBD and increased dramatically with increasing DBD. The dimer complex was slightly more abundant at low concentrations but became less abundant than the tetramer at higher DBD concentrations. Both complexes were abolished by an excess of unlabeled homologous oligonucleotide.

A similar experiment was performed with rat liver GR (Fig. 9B). The pattern of complex formation with GRE A up was compared with the pattern obtained with consensus GRE. In both cases, retarded complexes were observed at the lowest concentration, and their amounts increased with increasing GR concentration. As already observed, both monomer and dimer complexes were formed with consensus GRE, but the ratio of dimer to monomer increased as a function of GR concentration. In the case of GRE A up, the retarded probe was associated with a low-mobility complex similar to the one obtained with GRE A. Interestingly, this complex appeared at the lowest concentration of receptor, and very low amounts, if any, of the GR monomer or dimer complexes were formed. This experiment shows that the formation of the tetramer complex is highly cooperative, since no detectable intermediate complexes are formed even at very low GR concentrations. A similar conclusion could be drawn in the case of GRE A-GR complex formation (Fig. 3A).

To further confirm the sequences involved in GR binding, methylation interference experiments were performed. The labeled DNA fragment was partially methylated by DMS

lanes) and of the GR-GRE A up complex are shown to the left. (C) Semilog plot of the migration of bands 1 to 4 in panel A (GRE A) as a function of the molecular weight of complexes predicted to contain one to four molecules of DBD, respectively, with one molecule of oligonucleotide.

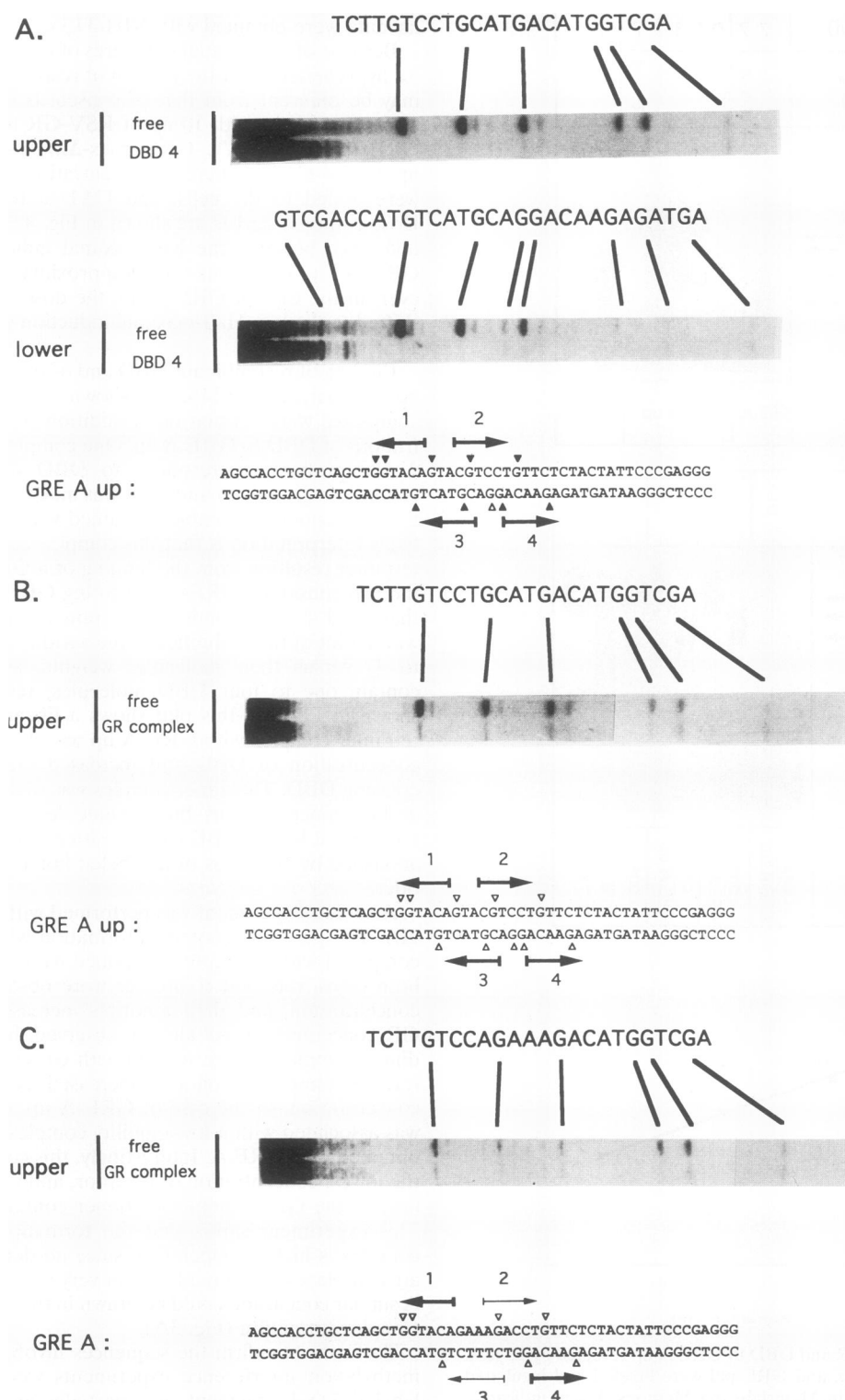


FIG. 10. DMS interference of receptor binding to GRE A or GRE A up. The oligonucleotides used are 52 bp long and comprise the GRE A or the GRE A up sequence flanked by 16 bp on each side. The flanking sequences are the natural sequences flanking GRE A in the cAspAT promoter. Probes were labeled on the upper or lower strand at the 5' end (phosphorylation with ^{32}P) and treated with DMS. Binding reaction, gel processing, and band isolation were done as described in Materials and Methods. The receptor was either GR DBD (A) or baculovirus-produced full-length GR (B and C), and the substrate was either GRE A up (A and B) or GRE A (C). In each case, the lowest-mobility complex was analyzed. The sequences are shown above the autoradiograms, and the G's which interfere with binding upon methylation are indicated. A summary of the data (some of which are not shown) is presented below each autoradiogram. Filled arrowheads indicate complete interference of the G upon methylation, and open arrowheads indicate partial interference.

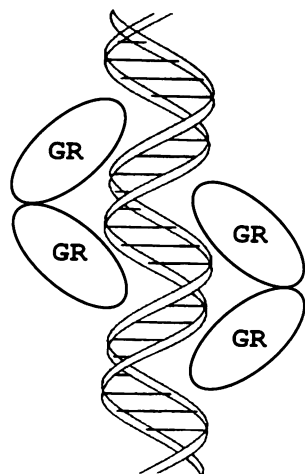


FIG. 11. Model representing the binding of a GR tetramer to GRE A or GRE A up. Two GR dimers bind to overlapping sites on the DNA. Each dimer is represented on one side of the DNA double helix, in agreement with the crystallographic structure of the DBD binding to a GRE (20). The distance between the centers of the two GRES constituting GRE A or GRE A up is 5 bp or half of a helix turn, which may allow the two GR dimers to bind to opposite sides of the DNA helix.

treatment, then allowed to bind to either GR-DBD or recombinant full-length GR, and used in EMSA. The resulting low-mobility complexes and free DNA were purified, cleaved by alkaline hydrolysis, and analyzed on sequencing gels. In protection assays using GR DBD and GRE A up as the substrate, the G residues present in all four half-sites are shown to be essential for the formation of the low-mobility complex (Fig. 10). When full-length GR was used in the protection assay, again all G residues of the four half-sites were required for DNA-GR complex formation. However, the protection was not as complete as that observed in the case of the DBD, suggesting some heterogeneity in the GR-GRE A up complex. This heterogeneity could be due to the fact that some complexes including an oligonucleotide with one methylated G may be stabilized by the strong cooperativity of the binding of the two GR dimers. A similar observation was made with respect to GR-GRE A interaction. In this case, the methylation of the G residues in half-sites 2 and 3 interfered even less with formation of the GR-DNA complex, but interference was nevertheless significant for the G residues of half-site 2.

DISCUSSION

In this study, we have characterized a novel 20-bp GRE located upstream of the cAspAT gene promoter (GRE A), composed of two overlapping imperfect GRES (called GRE 1-2 and GRE 3-4). These 15-bp elements are arranged in such a way that their centers are separated by 5 bp or half of a DNA helix turn. Each element consists of a highly conserved half-site (site 1 or 4) and a poorly conserved half-site (site 2 or 3) and is inactive by itself in a functional assay. The integrity of the whole structure is required to confer glucocorticoid inducibility.

DNase I footprinting experiments have shown that the GR binds to the GRE A sequence. Interesting properties of this binding were obtained from EMSAs. The complex formed between the GR and GRE A has a lower mobility than the one expected for a GR monomer or a GR dimer complex. Several

observations suggest that the complex is composed of two dimers forming a functional GR tetramer bound to DNA, as illustrated in Fig. 11. (i) This complex is formed in purified preparations of the rat liver GR or of recombinant full-length receptor. Furthermore, recombinant GR-DBD forms several complexes with GRE A, two of which migrate more slowly than the DBD dimer-DNA complex. The migration of these complexes is compatible with that of DBD trimer and DBD tetramer complexes. It is highly unlikely that these purified preparations of different forms of the receptor from totally different origins are contaminated by a protein that would retard the migration of the GR-GRE A complex. Instead, we believe that the receptor itself is responsible for this lower mobility. (ii) The sequence of GRE A is compatible with the presence of four GRE half-sites, two of which are highly conserved. Mutations that inactivate one or several half-sites yield the expected binding patterns in EMSA, i.e., either no binding or formation of monomer or dimer complexes. In all cases, the specific low-mobility complexes are lost. Furthermore, all of these mutants are inactive, demonstrating that a single GR dimer is not sufficient for activity. A particularly interesting mutant is the one in which the conserved residues in the imperfect half-sites 2 and 3 are replaced. In this case also, the binding of GR is very poor and the mutant is inactive. This finding suggests that the highly conserved half-sites are not the only ones required for efficient binding. (iii) Modification of the GRE A sequence, which is intended not to inactivate the responsive unit but rather to superactivate it, gave the expected results. Indeed, in GRE A up, the imperfect half-sites (2 and 3) have been modified in order to increase their affinity for GR, so that the resulting unit consists of two overlapping highly conserved GRE elements. This modified unit binds the GR with a higher affinity than GRE A and forms a complex with a similar mobility in EMSA. Furthermore, when binding of the DBD to GRE A up was evaluated, the low-mobility complex (band 4) was much more abundant than the one obtained with GRE A. Methylation protection experiments showed that all of the G residues of the four half-sites are required for the formation of the low-mobility DBD-GRE A up complex. Finally, functional assays showed that GRE A up conferred glucocorticoid inducibility with high efficiency, and compared with GRE A or consensus GRE, it decreased by approximately 1 order of magnitude the concentration of dexamethasone required to attain half-maximal induction. Thus, both binding and functional assays argue in favor of GR tetramer formation at the GRE A sequence. The predictions of this model, with respect to base modifications that would inactivate or superactivate the unit, have been experimentally confirmed.

We were initially surprised by the discrepancy between the relatively efficient binding of the DBD as a dimer to the mut 4 oligonucleotide and the inefficiency of this sequence in a functional assay. In this mutant, half-site 4 has been inactivated, which leaves GRE 1-2 as a putative responsive element. This element is inactive in a transfection assay mostly because half-site 2 is not perfect, as it includes an A instead of a T at position 3 (27). In fact, this half-site is more similar to an ERE half-site than to a GRE half-site. In contrast, half-site 1 is a typical GRE left half-site (2, 6). Thus, GRE 1-2 is a putative hybrid element. Interestingly, some of these hybrid elements have been studied by Truss et al. (37). One of the findings of that study which may be relevant to ours is that one of the hybrid elements (called AC-TT) could bind the progesterone receptor but was inactive in a functional assay. Therefore, the binding of a receptor as a dimer to an element is not sufficient to confer inducibility by the hormone. We hypothesize that a

similar mechanism may account for the observations made here on GRE 1-2.

One striking property of the pattern of GR binding to GRE A or to GRE A up is the poor representation of intermediate complexes such as monomer or dimer complexes (Fig. 9B). Even at the lowest receptor concentrations, the slowly migrating complex was predominant. This is particularly clear for GRE A up, which gives complexes migrating as relatively sharp bands (Fig. 9B). Such a pattern is typical of a highly cooperative binding reaction. Interestingly, the pattern of DBD binding is different. In the case of GRE A, the addition of increasing concentrations of DBD resulted first in the appearance of intermediate complexes, and then at the highest concentrations of DBD, a low-mobility complex appeared but constituted only a fraction of the bound DNA. Thus, the binding reaction with DBD displays much less cooperativity than with GR. In the case of GRE A up, only one intermediate complex corresponding to the DBD dimer can be detected. The absence of the other intermediate complexes is due to the fact that since all half-sites are highly conserved, both GRE 1-2 and GRE 3-4 interact with the DBD like the consensus GRE, that is, under the conditions used, mostly as a dimer (consensus GRE; Fig. 9A). Our conclusion is that the interactions yielding cooperative binding of GR as a tetramer to DNA require the presence of receptor domains outside the DBD.

There are several examples of cooperative binding of receptor dimers to adjacent hormone-responsive elements. Two imperfect EREs are found in tandem in the *Xenopus* vitellogenin B1 gene promoter (23). Each element by itself is inactive, but both elements in tandem mediate the hormonal response. This correlates with receptor dimers binding cooperatively to the whole sequence. The latter observation was questioned by a study of Ponglikitmongkol et al. (31). These authors found that in some constructs, EREs stimulated transcription synergistically in a stereoalignment-dependent manner but bound the receptor noncooperatively. In the case of the progesterone receptor, cooperative binding to adjacent GRE and progesterone-responsive element may account for the synergistic induction of a reporter gene (38). Another study led to the same conclusions for the GR (33). In the latter study, the half-life of receptor binding to duplicated GREs was shown to be 20-fold longer than that of the receptor dimer binding to a single GRE. The effect of the distance between the centers of these GREs on the stability of the GR-DNA complex was evaluated. Stability was highest when the centers of the GREs were separated by 2 or 3 helix turns but was only slightly decreased (20 to 25%) when the distance was near 2.5 DNA helix turns instead of 2. Thus, a considerable degree of cooperativity remained when the receptor dimers were bound on opposite sides of the helix. This could be explained either by a distortion of the DNA structure or by an important flexibility of the receptor molecule. The case of GRE A and GRE A up described here represents a novel type of cooperative interaction. The distance between the centers of the individual GREs is of half of a helical turn, and therefore the receptors bind to the DNA on opposite sides of the DNA helix. Nevertheless, a strong cooperativity is observed despite the fact that the two elements are overlapping. It is not clear at this stage whether the cooperativity observed with overlapping elements has the same structural basis as that observed with adjacent elements.

A negative GRE has been identified in the pro-opiomelanocortin gene promoter (10). Recently, it was demonstrated that the GR binds with high affinity to this element as a trimer, the DNA sequence being sandwiched between a GR dimer on one side of the helix and a GR monomer on the other (8, 9). In contrast to GRE A and GRE A up, this element does not

activate transcription despite receptor binding. Although it is clearly involved in glucocorticoid repression of the POMC gene promoter, the actual mechanism of such repression has not been elucidated. Therefore, the GR does not bind to target DNA elements solely as a homodimer. Depending on the sequence, it can also bind as a trimer or as a tetramer as shown here.

The significance of those observations is strengthened by the fact that they originate from naturally occurring sequences. Binding of the GR as a multimer to compact DNA sites may thus prove to be physiologically relevant. Although it is not yet possible to assign a specific function for each multimer, the experimental observations to date suggest that homodimers are the classical activators of transcription, as well as tetramers formed either on adjacent or overlapping sites as shown here. In one example, trimers may be involved in repression, while monomers could repress some promoters by interacting with other transcription factors. We can also speculate that tetramers may have properties different from those of dimers in activating transcription with respect to both sensitivity to different hormonal concentrations (or activated receptors; Fig. 8) and discrimination between receptors of the same family. Experimental evidence supporting the latter point has been obtained for adjacent GREs (32).

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